H. pylori-Induced DNA Strand Breaks Are Introduced by Nucleotide Excision Repair Endonucleases and Promote NF- B Target Gene Expression

Hartung, Mara L ; Gruber, Dorothea C ; Koch, Katrin N ; Grüter, Livia ; Rehrauer, Hubert ; Tegtmeyer, Nicole ; Backert, Steffen ; Müller, Anne

Abstract: The human bacterial pathogen Helicobacter pylori exhibits genotoxic properties that promote gastric carcinogenesis. H. pylori introduces DNA double strand breaks (DSBs) in epithelial cells that trigger host cell DNA repair efforts. Here, we show that H. pylori-induced DSBs are repaired via error-prone, potentially mutagenic non-homologous end-joining. A genome-wide screen for factors contributing to DSB induction revealed a critical role for the H. pylori type IV secretion system (T4SS). Inhibition of transcription, as well as NF- B/RelA-specific RNAi, abrogates DSB formation. DSB induction further requires 1-integrin signaling. DSBs are introduced by the nucleotide excision repair endonucleases XPF and XPG, which, together with RelA, are recruited to chromatin in a highly coordinated, T4SS-dependent manner. Interestingly, XPF/XPG-mediated DNA DSBs promote NF- B target gene transactivation and host cell survival. In summary, H. pylori induces XPF/XPG-mediated DNA damage through activation of the T4SS/ 1-integrin signaling axis, which promotes NF- B target gene expression and host cell survival.

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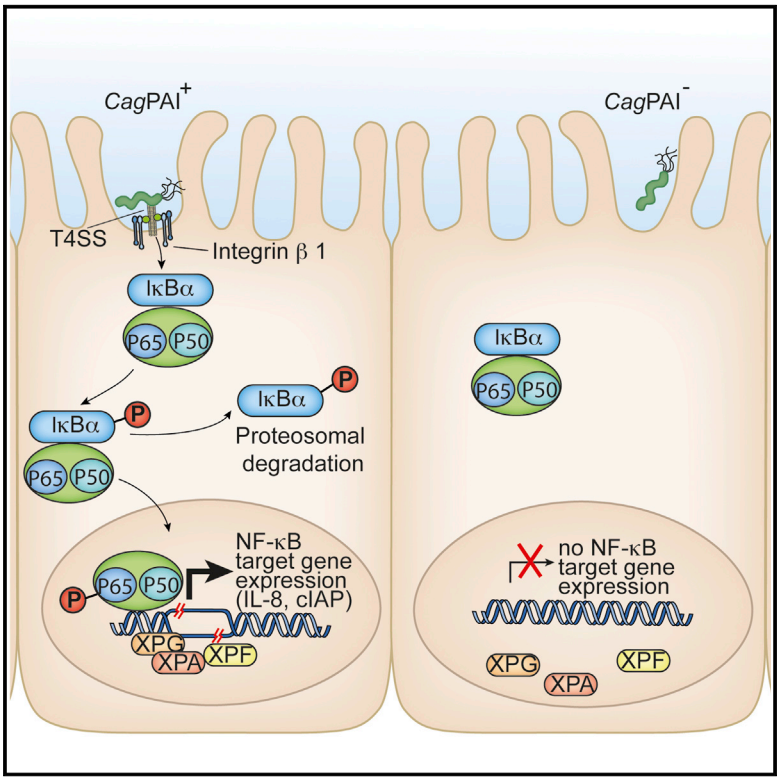
Graphical Abstract



Highlights

- *H. pylori* type IV secretion system is required for DNA double strand breaks (DSBs)
- *H. pylori*-induced DSBs are repaired via error-prone non-homologous end-joining
- DSBs are introduced by the nucleotide excision repair endonucleases XPF and XPG
- XPF/XPG-mediated DNA DSBs promote NF-κB target gene transactivation

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In Brief

In this study, Hartung et al. show that *H. pylori* introduces DNA double strand breaks (DSBs) in a type IV secretion system (T4SS)-dependent manner. The inhibition of transcription and NF-κB/RelA-specific RNAi abrogates DSBs. DSBs are introduced by XPF/XPG endonucleases and are required for NF-κB target gene activation and cell survival.

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SUMMARY

The human bacterial pathogen *Helicobacter pylori* exhibits genotoxic properties that promote gastric carcinogenesis. *H. pylori* introduces DNA double strand breaks (DSBs) in epithelial cells that trigger host cell DNA repair efforts. Here, we show that *H. pylori*-induced DSBs are repaired via error-prone, potentially mutagenic non-homologous end-joining. A genome-wide screen for factors contributing to DSB induction revealed a critical role for the *H. pylori* type IV secretion system (T4SS). Inhibition of transcription, as well as NF-κB/RelA-specific RNAi, abrogates DSB formation. DSB induction further requires β1-integrin signaling. DSBs are introduced by the nucleotide excision repair endonucleases XPF and XPG, which, together with RelA, are recruited to chromatin in a highly coordinated, T4SS-dependent manner. Interestingly, XPF/XPG-mediated DNA DSBs promote NF-κB target gene transactivation and host cell survival. In summary, *H. pylori* induces XPF/XPG-mediated DNA damage through activation of the T4SS/β1-integrin signaling axis, which promotes NF-κB target gene expression and host cell survival.

INTRODUCTION

Multiple species of pathogenic bacteria have been shown in recent years to damage the nuclear DNA of their host cells, often resulting in particularly hazardous DNA double strand breaks (DSBs) (Chumduri et al., 2013; Hanada et al., 2014; Nougayrède et al., 2006; Samba-Louaka et al., 2014; Toller et al., 2011). The phenomenon was first reported for *Escherichia coli* strains expressing peptide-polyketide genotoxins, which induce DNA DSBs and trigger the canonical mammalian DNA damage and repair response, leading to cell-cycle arrest and ultimately apoptotic cell death (Nougayrède et al., 2006). The carcinogenic bacterial pathogen *Helicobacter pylori*, which causes chronic gastritis and is the most important risk factor for the development of peptic ulcer disease and gastric cancer, also exhibits genotoxic activity (Hanada et al., 2014; Toller et al., 2011). Exposure of gastric epithelial cells to *H. pylori* induces DNA fragmentation in a contact-dependent manner that is detectable by pulsed field gel electrophoresis (PFGE) and can be visualized by high resolution microscopy of metaphase chromosomes; the DSB repair factor p53 binding protein 1 (53BP1) and others are recruited to the sites of DSBs and, together with the phosphorylation of histone H2A variant X (γH2AX), indicate the initiation of DSB repair by the host cell repair machinery (Toller et al., 2011). Several intracellular bacterial pathogens also trigger DNA DSBs; most notably, *Chlamydia trachomatis* induces DSBs through the production of reactive oxygen species but inhibits the downstream repair and cell-cycle checkpoint activation that typically follow DSB induction (Chumduri et al., 2013). A similar scenario has been reported for *Listeria monocytogenes*, which also triggers DNA DSBs but actively prevents the DNA damage response (DDR) in a manner depending on listeriolysin O-mediated degradation of the DNA damage sensor MRE11 (Samba-Louaka et al., 2014). Both *Listeria* and *Chlamydia* benefit from preventing the DDR, as they continue to replicate in their actively cycling host cells despite massive DNA damage (Chumduri et al., 2013; Samba-Louaka et al., 2014). Whereas the induction of DNA DSBs thus seems to be a pathogenetic trait shared by several intra- and extracellular, intestinal, and extraintestinal pathogens, the mechanisms employed by the bacteria to induce DSBs and deal with the subsequent host cell DDR remain poorly understood.

Here, we have investigated the mechanistic basis of *H. pylori*-induced DNA DSBs and have elucidated key host and bacterial factors contributing to DSB induction. By screening an *H. pylori* transposon library for mutants lacking the ability to induce host cell DNA DSBs, we have identified the *cag* pathogenicity island (*cag*PAI)-encoded type IV secretion system (T4SS) as being critically involved in *H. pylori*-induced DNA damage. We have further found active transcription and the T4SS/β1 integrin-induced activation of nuclear factor kappa B (NF-κB) to be

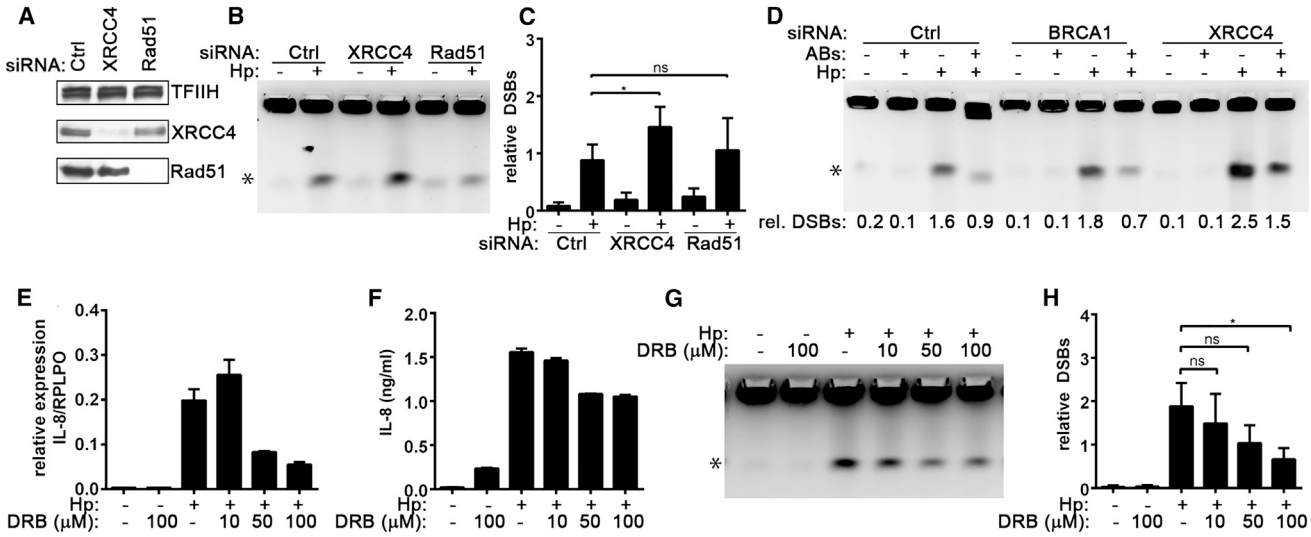


Figure 1. *H. pylori* Induces Transcription-Dependent Host Cell DNA Double Strand Breaks That Are Repaired by the Non-homologous End-Joining Pathway

(A–C) AGS cells were transfected with siRNAs targeting XRCC4 or Rad51 or with an unspecific control siRNA 72 hr prior to infection with *H. pylori* strain G27 (MOI 50). Cells were harvested 6 hr post-infection (p.i.) and subjected to (A) western blotting for XRCC4 and Rad51 (and TFIIH as loading control) to verify the knockdown efficiency and (B and C) PFGE to assess the formation of DNA DSBs. A representative PFGE image is shown in (B); means ± SD of three experiments are shown in (C). Asterisks indicate fragmented DNA.

(D) AGS cells were transfected with siRNAs targeting XRCC4 or Rad51 or a control siRNA 24 hr prior to infection with *H. pylori* strain G27 (MOI 50, 48 hr). Bacteria were eliminated by antibiotic treatment (ABs) for 24 hr, after which all cells were harvested and subjected to PFGE. A representative PFGE image of three is shown along with its quantification.

(E–H) AGS cells were pretreated for 2 hr with increasing concentrations of the transcription inhibitor DRB prior to infection with *H. pylori* strain G27 (MOI 50). Cells and supernatants were harvested 4 hr p.i. and assessed for IL-8 transcript levels by qRT-PCR (E), IL-8 secretion by ELISA (F), and PFGE for DSB formation (G and H). A representative experiment of three is shown in (E)–(G); means ± SD of three experiments are shown in (H).

See also Figure S1.

important prerequisites of DSB induction and have identified the nucleotide excision repair (NER) endonucleases XPF and XPG as critical enzymes introducing the strand breaks. XPF/XPG-mediated DSBs serve to amplify NF-κB target gene expression and promote host cell survival. In summary, we describe a novel mechanism of pathogen-induced, transcription-dependent DNA DSB induction and highlight the importance of this mechanism for the amplification of NF-κB target gene transactivation and the survival of *H. pylori*-infected cells.

RESULTS

H. pylori-Induced DNA DSBs Are Repaired via Non-homologous End-Joining Rather Than Homologous Recombination and Are Incurred during Active Transcription

Having shown earlier that *H. pylori* induces DNA DSBs in the nuclear genome of its target cells, we asked whether the infected cell is capable of addressing the damage and set out to identify the predominant repair mechanism. To this end, we silenced the expression of key components of the non-homologous end-joining (NHEJ) and homologous recombination (HR) DSB repair pathways by small interfering RNAs (siRNAs) prior to infection of AGS gastric epithelial cells with *H. pylori* G27. Interestingly, the siRNA-mediated knockdown of the NHEJ pathway protein XRCC4, which links DNA ligase IV to the damaged DNA ends, resulted in a significant accumulation of fragmented DNA as detected by PFGE (Figures 1A–1C). In contrast, the parallel knockdown of the HR protein Rad51, which is involved in homology searching and strand pairing, did not affect the amount of fragmented DNA generated during *H. pylori* infection (Figures 1A–1C). The efficiency of both siRNAs was high and comparable (Figure 1A); the physiological consequences of both knockdowns could further be confirmed in the setting of etoposide-induced DNA DSBs, which accumulated in the absence of XRCC4 and, to a lesser extent, Rad51 (Figure S1A). siRNAs targeting additional components of the NHEJ pathway (i.e., 53BP1 [Figure S1B], DNA-PK and DNA ligase IV [data not shown]) confirmed that this pathway is key to the repair of *H. pylori*-induced DSBs.

The above results suggest that NHEJ is required for the continuous ("steady-state") repair of DSBs incurred during *H. pylori* infection. However, DSBs are not repaired completely unless the bacteria are killed by antibiotics and cells are allowed to repair their DNA in the absence of live bacteria. Efficient repair is detectable as early as 24 hr after addition of the antibiotics and is completed after ~48 hr (Figure 1D; Figure S1C). The siRNA-mediated knockdown of XRCC4 or 53BP1, but not the HR repair factor BRCA1, reduced the cells' repair capability (Figure 1D; Figure S1B), further supporting the notion that *H. pylori*-induced DSBs are preferentially addressed by the NHEJ pathway.

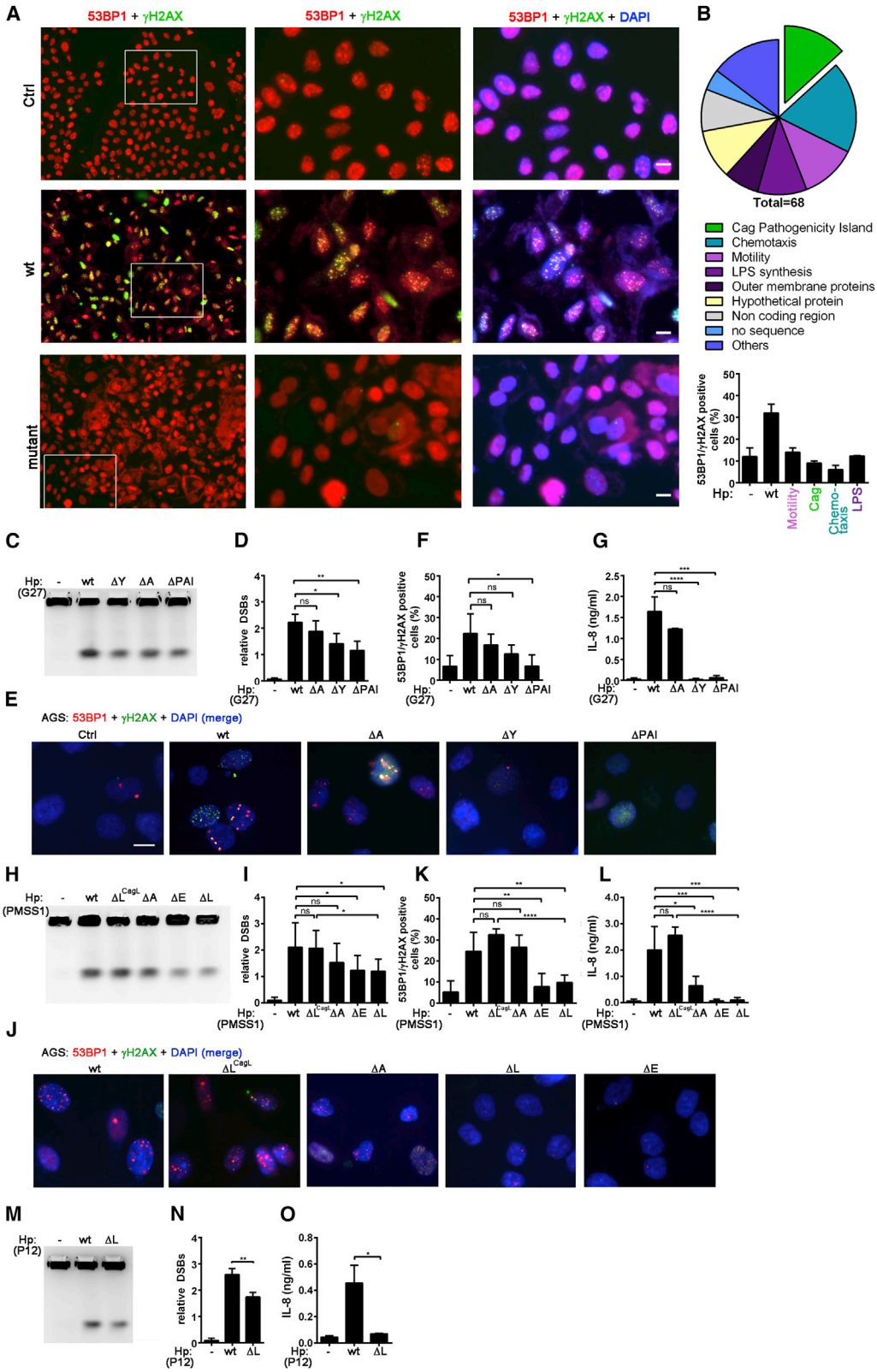
As DSBs often occur at transcribed regions of the genome and transcription-associated DNA damage is an important source of mutations (Jinks-Robertson and Bhagwat, 2014), we asked whether the pharmacological inhibition of transcription would affect *H. pylori*-induced DNA DSB induction. We treated infected cells with increasing concentrations of the transcription initiation inhibitor 5,6-dichloro-1-β-D-ribofuranosyl-1H-benzimidazole (DRB), which strongly reduced expression of *H. pylori*-induced IL-8 (encoded by a canonical, *cag*PAI-dependent *H. pylori* target gene in AGS cells) at the transcript and protein levels (Figures 1E and 1F), but did not affect *H. pylori* infectivity as judged by western blotting for phospho-CagA (Figure S1D). Interestingly, treatment with the transcription inhibitor dose-dependently reduced the formation of *H. pylori*-induced DNA DSBs (Figures 1G and 1H), indicating that active transcription is indeed a key prerequisite of this type of DNA damage.

A Genome-wide Screen for *H. pylori* Genes Affecting DSB Induction Reveals a Critical Contribution of the T4SS

We next set out to identify *H. pylori* factors involved in DSB induction. To this end, we obtained a transposon mutant library that had been used previously for a variety of in vitro and in vivo screens (Salama et al., 2004). Having shown previously that the DNA damage induced by *H. pylori* results in the focal accumulation of host cell repair factors such as 53BP1 and the phosphorylation of H2AX at sites of DNA DSBs, we opted for an immunofluorescence microscopy-based screening readout. Cells were grown in 96-well format, infected with 2,000 individual transposon mutants, stained with antibodies specific for 53BP1 and phosphorylated H2AX (γH2AX), and visually inspected with regard to foci formation (Figure 2A). Mutants exhibiting a >50% reduction in the number of foci-positive nuclei were subjected to a second round of screening and examined with respect to their viability and motility. We identified 68 mutants with defects in inducing 53BP/γH2AX foci that could be mapped to 36 different genomic loci (Table S1; Figure 2B). Five categories of mutants were identified repeatedly; these had insertions in genes involved in lipopolysaccharide (LPS) biosynthesis, chemotaxis, and flagellar motility, as well as in genes encoding outer membrane proteins and *cag*PAI proteins (Table S1). All chemotaxis and motility hits are likely attributable to a lack of binding to target cells, a known prerequisite of *H. pylori*-induced DNA damage (Toller et al., 2011). The mutants lacking LPS biosynthesis genes are likely to exhibit a defect in assembling a functional T4SS as published previously (Chang et al., 2011). Strikingly, a total of 12% of all hits of the screen affected genes encoded on the *cag*PAI. In particular, transposon insertions were identified in the genes encoding CagE, H, M, X, Y and Z, which share the common feature of being required for IL-8 secretion (Backert et al., 2015; Barrozo et al., 2013; Fischer et al., 2001; Shaffer et al., 2011). We next tested isogenic gene deletion mutants lacking the entire island (Δ*cag*PAI), individual *cag*PAI genes essential for IL-8 secretion (Δ*cagE* and Δ*cagY*), or the *cag*PAI-encoded T4SS adhesin CagL and effector CagA (Δ*cagL* and Δ*cagA*) in up to three different strain backgrounds with respect to their ability to induce repair foci and fragmented DNA as determined by microscopy and PFGE; all mutants were further examined for their ability to induce IL-8 secretion (Figures 2C–2O). Mutants lacking the entire *cag*PAI, or CagE, CagY, or CagL, exhibited a strongly reduced ability to induce DNA damage as determined by both readouts, whereas a Δ*cagA* mutant induced DNA damage at close to wild-type levels (Figures 2C–2O). The DNA-damaging capacity of each mutant correlated well with its ability to induce IL-8 secretion as determined by ELISA (Figures 2C–2O) as well as its ability to induce the transcription of the NF-κB target gene cIAP (Figure S2A), but could not be attributed to lack of adhesion to target cells (Figures S2B and S2C; data not shown). The defect of the CagL mutant could be restored by the genetic complementation of CagL in two strain backgrounds (Figures 2H–2J; Figures S2D–S2F). Treatment of AGS cells with recombinant CagL, but not recombinant CagA, induced low levels of DNA damage (data not shown); however, the amounts of rCagL required to observe effects by PFGE were rather high at 50 and 100 μg/ml and therefore do not support definitive conclusions on a more important role of CagL over other Cag proteins in triggering DNA damage. The combined data suggest that the *cag*PAI-encoded T4SS drives *H. pylori*-induced DSB formation and the subsequent recruitment of repair factors to sites of DNA damage.

The Formation of *H. pylori*-Induced DSBs Depends on NF-κB p65/RelA

We and others have shown that the *H. pylori* T4SS induces NF-κB activation and IL-8 production by consecutively activating integrin α5β1, JNK, and ERK in a CagL-dependent manner (Gorrell et al., 2013; Kwok et al., 2007). We confirmed that Δ*cagE* and Δ*cagL* mutants are incapable of activating NF-κB as determined by western blotting for phospho-RelA and phospho-IκBα (Figures 3A and 3B) and also fail to induce IL-8 (Figure 2). Speculating that NF-κB activation might be required for the induction of *H. pylori*-specific DNA DSBs, we silenced the expression of p65/RelA by RNAi (>90%; Figure 3C). Interestingly, the induction of DSBs was greatly reduced by silencing of RelA in AGS cells (Figures 3D and 3E), indicating that NF-κB p65/RelA is crucially involved in *H. pylori* DSB induction. As expected, RelA depletion strongly reduced the *H. pylori*-induced production and secretion of IL-8 (Figure 3F). The same phenotype was observed in another cell line, U2OS, which is readily infected by *H. pylori* (Figures 3G–3J). Similar results were further obtained with a dominant-negative mutant of the inhibitor of NF-κB, IκBα, lacking two serines in positions 32 and 36 that are required for its phosphorylation and subsequent proteasomal degradation (Van Antwerp et al., 1996). The overexpression of dominant-negative IκBα reduced both IL-8 production and DSB induction (Figures 3K–3N). As mentioned earlier, integrin α5β1 is believed to serve as the epithelial cell receptor for CagL (Kwok et al., 2007), as well as another component of the T4SS, CagY, and the translocated effector CagA (Jiménez-Soto et al., 2009). Consequently, the loss of β1 integrin or its inhibition by blocking antibodies results in defective CagA translocation and NF-κB activation (Gorrell et al., 2013; Jiménez-Soto et al., 2009; Kwok et al., 2007). To address whether β1 integrin is also required for DNA DSB induction, we infected AGS cells in which β1 integrin was stably knocked down due to small hairpin



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RNA (shRNA) expression; these cells showed a strongly decreased sensitivity to *H. pylori*-induced DSBs, as well as diminished production of IL-8 (Figures 3O–3R). In contrast, the siRNA-mediated depletion of Nod1, a cytoplasmic sensor for peptidoglycan, did not affect DSB induction (Figures S3A–S3C).

Among many other targets, NF-κB regulates the expression of activation-induced cytidine deaminase (AID), an enzyme reported to be aberrantly activated in *H. pylori*-infected epithelial cells (Matsumoto et al., 2007). Speculating that *H. pylori*-induced NF-κB activity might lead to DSBs via AID activation, we examined a putative role of AID in our model of *H. pylori*-induced DSBs. We were not able to detect enhanced expression of AID upon *H. pylori* infection in AGS cells (Figure S3D), and the specific siRNA-mediated silencing of AID did not affect *H. pylori*-induced DSBs (Figures S3E and S3F). The combined results implicate the T4SS/β1 integrin/NF-κB signaling axis in *H. pylori*-induced DSB induction but rule out a critical contribution of Nod1 or aberrantly activated AID.

H. pylori-Induced DSBs Require the Activity of the NER Machinery

Two novel mechanisms have recently been described to facilitate the nuclear hormone receptor-regulated transactivation of target genes by introduction of DNA DSBs; one involves the NER endonuclease XPG (Le May et al., 2012; Le May et al., 2010), whereas the other requires the activity of topoisomerase 2B (TOP2B) (Haffner et al., 2010). The XPG endonuclease was shown to be recruited to the promoter regions of actively transcribed nuclear receptor target genes (Le May et al., 2010), where it facilitates transcription initiation by introducing DSBs and promoting DNA demethylation and gene looping (Le May et al., 2012). Silencing of XPG strongly reduced the formation of fragmented DNA upon *H. pylori* infection (Figures 4A–4C); a second cell line and a second, unrelated XPG-specific siRNA reproduced the phenotype (Figures S4A–S4F). siRNAs directed at two other components of the NER machinery, the recruitment factor XPA and the 3′ endonuclease XPF, phenocopied the effect of XPG depletion (Figures 4D–4I). In contrast, the silencing of an endonuclease functioning as part of the base excision repair machinery, apurinic endonuclease 1 (APE1), failed to affect *H. pylori*-induced DSBs in a measurable manner (Figures S4G–S4I).

Strikingly, we found that silencing of XPG, XPF, and XPA robustly reduced IL-8 secretion by *H. pylori*-infected cells (Figures 4J–4L), a phenotype that could not be attributed to effects of XP protein depletion on the upstream events of NF-κB activation (IκBα phosphorylation and degradation, RelA phosphorylation; Figure 4M–4O; Figures S4J–S4L). To examine whether the induction of DNA DSBs would also be observed with another inducer of IL-8 secretion, tumor necrosis factor α (TNF-α), we treated U2OS cells with increasing concentrations of the recombinant cytokine. No evidence of DNA DSBs could be obtained upon TNF-α treatment (Figure 4P), which efficiently activated NF-κB (Figure S4M). Furthermore, the knockdown of XPG had no discernible effect on TNF-α-induced IL-8 secretion (Figure 4Q), indicating that the DNA DSBs observed in *H. pylori*-infected cells are rather specifically associated with NF-κB activation during this host/pathogen interaction. Interestingly, the impaired expression and secretion of *H. pylori*-induced IL-8 in the absence of XP proteins is mirrored by the reduced expression of other NF-κB-dependent gene products, as shown for the cellular inhibitor of apoptosis protein, cIAP (Figure 4R).

To examine a possible contribution of TOP2B to *H. pylori*-induced DSBs, we treated infected cells with increasing concentrations of the TOP2B inhibitor ICRF. TOP2B inhibition had no effect on the amount of fragmented DNA in infected cells or on IL-8 production (Figures 4S–4U) or on *H. pylori* T4 secretion (Figure S4N), although the same treatment efficiently prevented etoposide-induced DSBs (Figures S4O and S4P). The combined results indicate that *H. pylori*-induced DNA DSBs require the XPG and XPF endonucleases, but not TOP2B, and that NF-κB target gene expression is enhanced by the activation of the NER complex.

NER Factors Are Recruited to Chromatin of *H. pylori*-Infected Cells in a T4SS-Dependent Manner and Promote Cell Survival

To study the kinetics of XP protein recruitment to the DNA of *H. pylori*-infected cells, we extracted chromatin from AGS cells at 1.5, 3, and 6 hr post-infection (p.i.) and subjected it to western blotting. The XPG endonuclease and XPA are recruited to chromatin within 1.5 to 3 hr of *H. pylori* exposure, and simultaneously with NF-κB RelA (Figures 5A and 5B). The coordinated recruitment of RelA, XPG and XPA to chromatin is dependent on CagL and thus on a functional T4SS; the restoration of CagL expression partially (albeit not statistically significant across multiple experiments) complements the phenotype (Figures 5C and 5D).

Having shown earlier that *H. pylori*-infected epithelial cells survive for long periods of time despite sustaining massive DNA

Figure 2. *H. pylori*-Induced DNA Damage Is Dependent on a Functional T4SS and CagL

(A and B) 2,000 transposon mutants generated in *H. pylori* NSH57 were individually assessed by visual inspection for their ability to induce the formation of nuclear 53BP1 (red)-, γH2AX (green)-positive repair foci; 68 mutants were confirmed in two rounds of screening to exhibit a >50% reduced ability to induce foci. Representative images are shown in (A) along with high-magnification insets (scale bars, 10 μm), and a cake diagram depicting the identified mutant categories along with a quantification of representative mutants is shown in (B).

(C–G) AGS cells were infected with wild-type *H. pylori* G27 or the indicated isogenic Cag deletion mutants (MOI 50). Cells were either harvested at 6 hr p.i. for PFGE (a representative PFGE image and means ± SD of five experiments are shown in C and D) or at 48 hr p.i. for 53BP1 and γH2AX staining (representative images are shown in E and means ± SD of five experiments are shown in F; scale bar, 10 μm). Supernatants of cells infected for 6 hr were assessed for IL-8 secretion by ELISA (means ± SD of five experiments are shown in G).

(H–L) AGS cells were infected with wild-type *H. pylori* PMSS1 or the indicated isogenic Cag deletion mutants (MOI 50) and processed as described for (C)–(G). Means ± SD of four experiments are shown in (I), (K), and (L); representative images are shown in (J).

(M–O) AGS cells were infected with wild-type *H. pylori* P12 or an isogenic CagL deletion mutant (MOI 50) and processed as described for (C)–(G). Means ± SD of three experiments are shown in (N) and (O).

See also Figure S2.

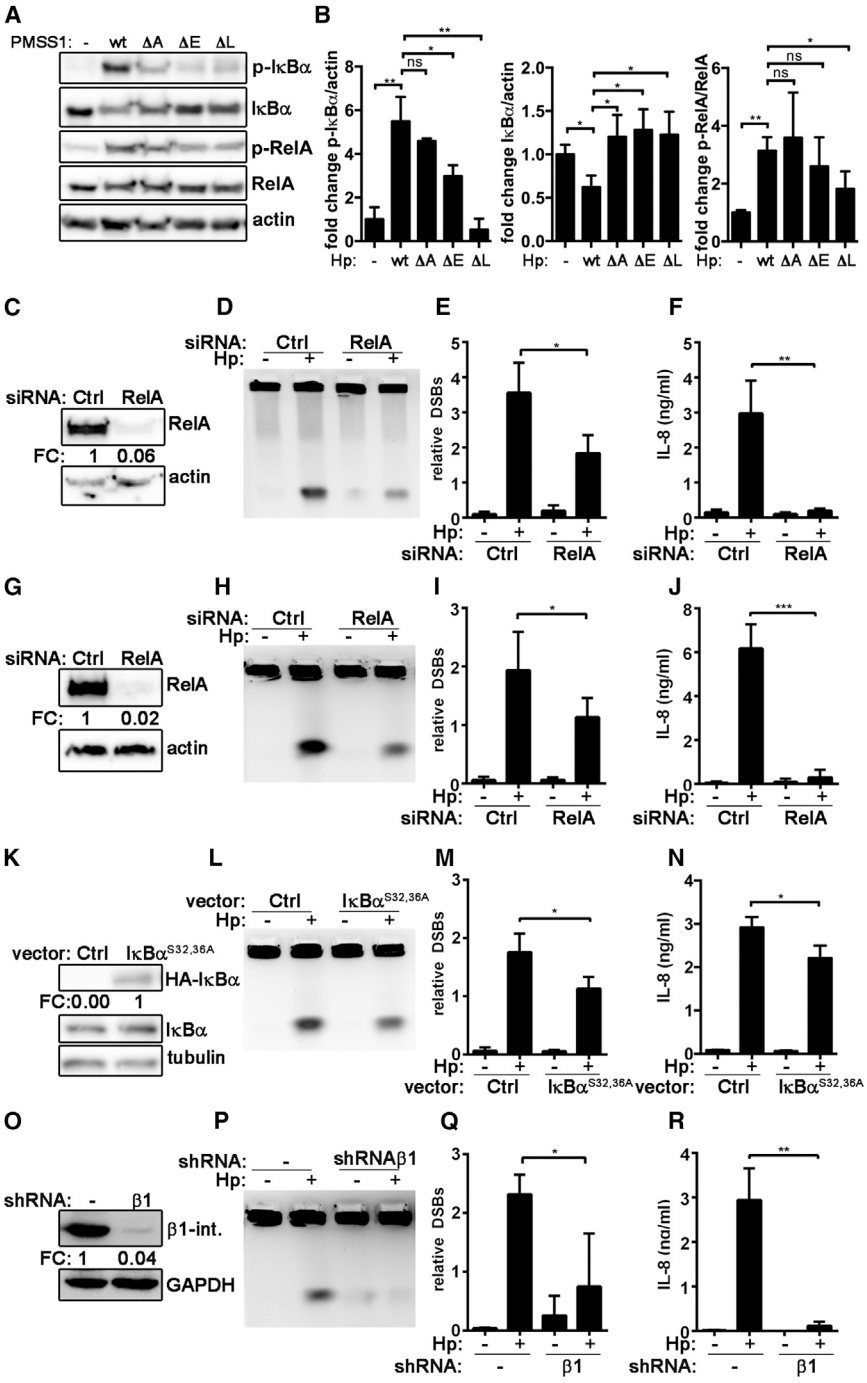

Figure 3. The Formation of *H. pylori*-Induced DNA DSBs Requires NF-κB RelA and β1 Integrin

(A and B) AGS cells were infected with wild-type *H. pylori* PMSS1 or the indicated isogenic Cag deletion mutants (MOI 50) and harvested 6 hr p.i. for western blotting using antibodies against the phosphorylated forms of RelA and IκBα, as well as the respective total proteins and actin. A representative western blot is shown in (A), along with the quantification (means ± SD) of three experiments in (B).

(C–J) AGS cells (C–F) and U2OS cells (G–J) were transfected with a RelA-specific siRNA or control siRNA 72 hr prior to infection with *H. pylori* strain G27 (MOI 50). Cells and supernatants were harvested 6 hr p.i. and subjected to western blotting for RelA to verify the knockdown efficiency (C and G; FC, fold change relative to control siRNA) and PFGE to assess DNA DSBs, respectively, and to IL-8 ELISA. Representative PFGE images are shown in (D) and (H); means ± SD of four (E and F) and five (I and J) experiments are shown.

(K–N) AGS cells were transfected with a vector encoding a dominant-negative mutant of IκBα or the empty vector 48 hr prior to infection with *H. pylori* strain G27 (MOI 50). Cells and supernatants were processed as described for (C)–(J); means ± SD of four experiments are shown in M,N.

(O–R) AGS cells stably expressing a β1-integrin-specific shRNA were infected with *H. pylori* strain G27 (MOI 50) and compared to control AGS cells. Cells and supernatants were processed as described for (C)–(J); means ± SD of three experiments are shown in (Q) and (R).

See also Figure S3.

damage (Toller et al., 2011), and that XPG/XPF-dependent DSBs promote NF-κB-specific (anti-apoptotic, e.g., of cIAP) gene expression, we speculated that XP proteins might contribute to cell survival in the context of *H. pylori* infection. As reported, no or only minimal cell death is induced by the infection alone in AGS cells (Figures 5E and 5F). Interestingly, silencing of XPA renders the cells more susceptible to apoptosis in the presence of live bacteria (Figures 5E and 5F). The combined results are consistent with the model that the T4SS/CagL-induced nuclear translocation and chromatin recruitment of NF-κB serves as a platform for the subsequent recruitment of XP proteins, which enhances anti-apoptotic gene expression and promotes cell survival.

DISCUSSION

In this study, we have addressed the molecular mechanism underlying the induction of DNA DSBs in *H. pylori*-infected cells and have identified critical bacterial as well as host cell factors contributing to the DNA damage. A genome-wide screen was designed to identify *H. pylori* transposon mutants lacking the ability to induce DSBs; strikingly, 12% of all transposon insertions mapped to genes of the *cag*PAI (*cagE*, *H*, *M*, *X*, *Y*, and *Z*) that had previously been implicated in T4SS assembly and/or NF-κB-dependent IL-8 production (Fischer et al., 2001; Shaffer et al., 2011). Isogenic gene-specific deletion mutants confirmed that a functional T4SS,

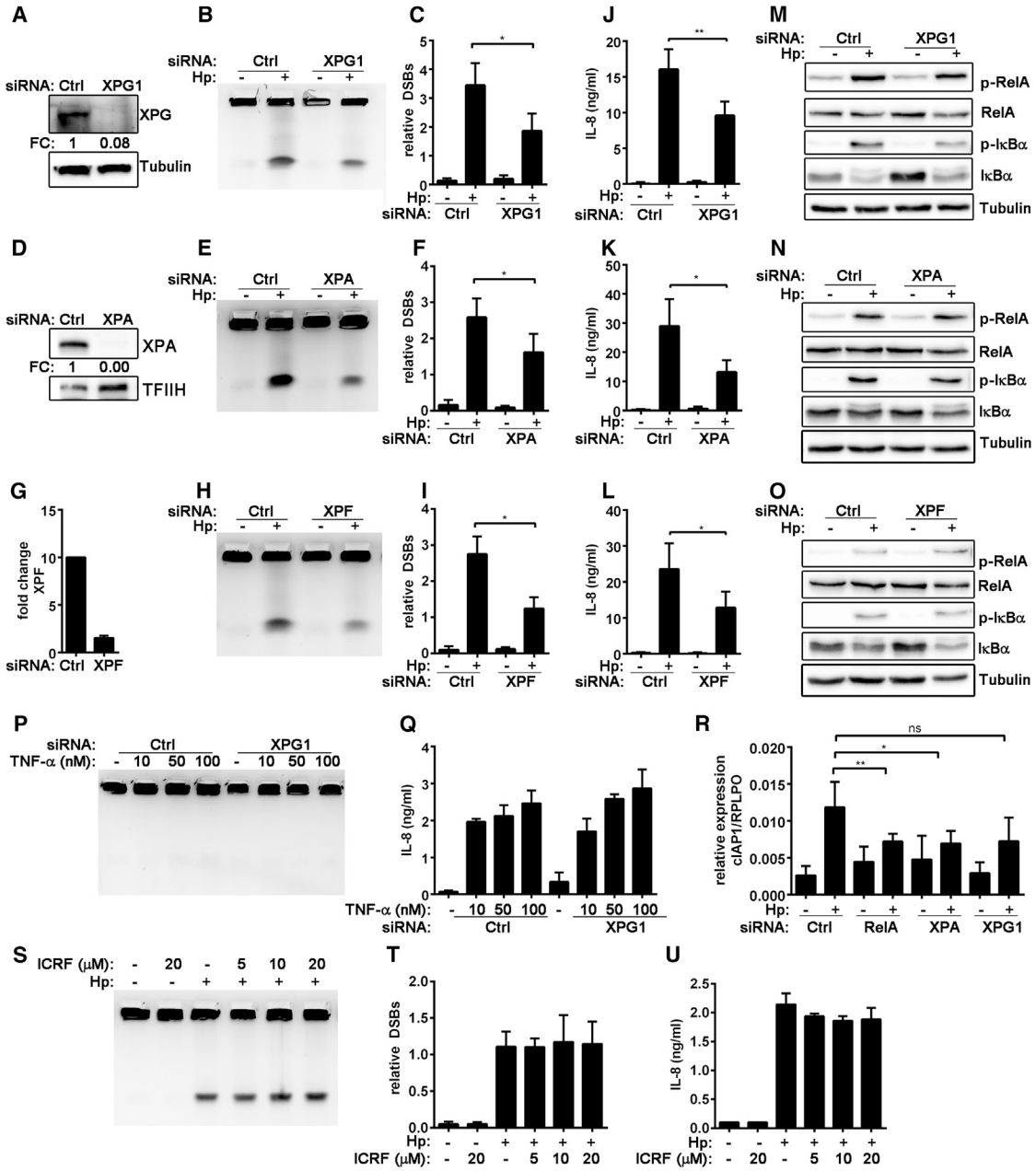

Figure 4. The Formation of *H. pylori*-Induced DNA DSBs Requires the NER Endonucleases XPG and XPF

(A–C) U2OS cells were transfected with an XPG-specific siRNA or control siRNA 72 hr prior to infection with *H. pylori* strain G27 (MOI 50). Cells were harvested 6 hr p.i. and subjected to (A) western blotting for XPG to verify the knockdown efficiency and (B and C) PFGE to assess DNA DSBs. A representative PFGE image is shown in (B); means ± SD of three experiments are shown in (C).

(D–F) U2OS cells were transfected with an XPA-specific siRNA or control siRNA and otherwise treated and processed as described for (A)–(C). Means ± SD of four experiments are shown in (F).

(G–I) U2OS cells were transfected with an XPF-specific siRNA or control siRNA and otherwise treated and processed as described for (A)–(C). Means ± SD of three experiments are shown in (I). Due to the lack of a suitable antibody, the knockdown efficiency of XPF had to be assessed by qRT-PCR (in G).

(J–L) Supernatants harvested from cells treated as described in (A)–(C), (D)–(F), and (G)–(I) were subjected to IL-8 ELISA. Pooled ELISA data for the same experiments as shown in (C), (F), and (I) (n = 3, 4 and 3) are shown as means ± SD.

(M–O) Extracts of cells treated as described in (A)–(C), (D)–(F), and (G)–(I) were subjected to western blotting using the indicated antibodies. Representative western blots are shown; see Figure S4 for the quantification of three independent experiments.

(P and Q) U2OS cells were treated for 6 hr with the indicated concentrations of TNF-α and subjected to PFGE (P) and IL-8 ELISA (Q). A representative experiment of three is shown.

(legend continued on next page)

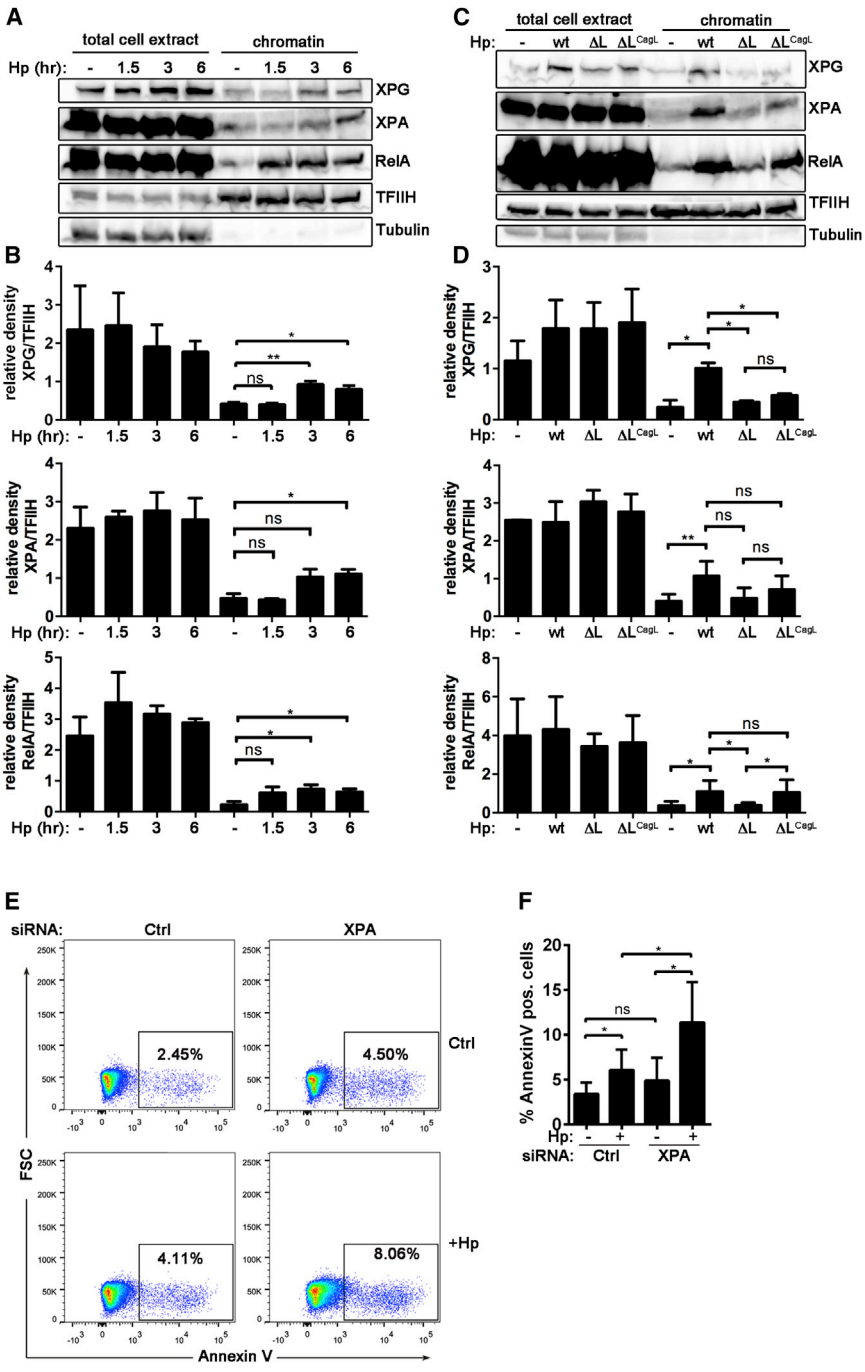

Figure 5. NER Factors Are Recruited to the Chromatin of *H. pylori*-Infected Cells along with RelA and Suppress Infection-Associated Apoptosis

(A and B) AGS cells were infected with *H. pylori* wild-type PMSS1 for 1.5, 3, and 6 hr and subjected to whole-cell extraction as well as chromatin enrichment. Total cell extract and chromatin were analyzed by western blotting using the indicated antibodies. A representative experiment is shown in (A) along with relative densities of XPG, XPA, and RelA (means ± SD) of three experiments in (B).

(C and D) AGS cells were infected with wild-type PMSS1, PMSS1Δ*cagL*, and PMSS1Δ*cagL*$^{CagL-}$ for 6 hr and processed as described in (A) and (B). A representative experiment is shown in (C) along with relative densities of XPG, XPA, and RelA (means ± SD) of three experiments in (D).

(E and F) AGS cells were transfected with XPA-specific or control siRNAs 48 hr prior to infection with *H. pylori* PMSS1 (MOI 50). Cells were harvested 24 hr p.i. and subjected to annexin V staining followed by flow cytometric analysis. Representative fluorescence-activated cell sorting plots are shown in (E), and means ± SD of six experiments are shown in (F).

and presumably its interaction with target cells via the CagL/β1 integrin axis, are required for efficient DSB induction. The results of our screen are in line with a recent publication demonstrating that both *cag*PAI-positive and *cag*PAI-negative strains can in principle induce DNA DSBs, but the former do so significantly more robustly (Hanada et al., 2014).

The T4SS, presumably via its adhesin CagL, is known to induce the nuclear translocation of NF-κB and target gene transactivation in gastric epithelial cells via β1 integrin signaling (Gorrell et al., 2013; Kwok et al., 2007); therefore, the dependence of *H. pylori*-induced DSBs on a functional T4SS, CagL, and β1 integrin is in line with a critical contribution of NF-κB RelA to DSB induction. Cells depleted of RelA or expressing dominant-negative IκBα are largely resistant to DSBs; interestingly, the activation of NF-κB by an unrelated trigger such as TNF-α fails to induce DSBs, suggesting that the phenomenon is specifically associated with *H. pylori*. It is conceivable that the specific signaling pathway activated (downstream of β1 integrin or of the TNF receptor) dictates whether DSBs are incurred or not; alternatively, the strength of the NF-κB-activating signal,

(R) U2OS cells were transfected with RelA-, XPA-, and XPG-specific siRNAs or control siRNA 72 hr prior to infection with *H. pylori* strain G27 (MOI 50). Cells were harvested 6 hr p.i. and subjected to qRT-PCR for cIAP. Means ± SD of three experiments are shown.

(S–U) U2OS cells were pretreated with the indicated concentrations of the TOP2B inhibitor ICRF for 2 hr prior to infection with *H. pylori* strain G27 (MOI 50). Cells and supernatants were harvested 4 hr p.i. and subjected to (S and T) PFGE to assess DNA DSBs and (U) IL-8 ELISA. A representative PFGE image is shown in (S) and means ± SD of two experiments are shown in (T) and (U).

See also Figure S4.

which is approximately ten times higher upon infection than upon TNF-α treatment, may affect the extent of DNA DSBs.

As our screen for *H. pylori* factors directly involved in DNA DSB induction failed to identify endonucleases or other DNA-modifying enzymes, we next asked which host cell enzyme(s) could be assuming this activity. One possible candidate was the apurinic endonuclease APE1, which introduces nicks at abasic sites that arise as a consequence of the excision of damaged bases; however, the siRNA-mediated silencing of APE1 failed to affect *H. pylori*-induced DNA DSBs. We next focused on the NER endonucleases XPG and XPF, mostly because these had previously been implicated in the transcription-associated DSBs found at the start sites of actively transcribed nuclear receptor target genes (Le May et al., 2010, 2012). Indeed, the RNAi-mediated depletion of XPG and XPF, as well as of the XP recruitment factor XPA, strongly reduced the induction of DSBs as determined by PFGE; this observation leads us to propose a model in which XPG and XPF are actively recruited to NF-κB-bound promoters, where they introduce closely spaced nicks in the sugar phosphate backbone of both strands to produce DSBs. As we used RNAi to deplete XP proteins rather than mutate their catalytic activity, our results do not formally rule out a non-enzymatic contribution of XPF and XPG to DNA DSB induction; however, as no such non-catalytic function has yet been assigned to the XP endonucleases, this scenario seems unlikely.

We next set out to determine the consequences of NER-induced DNA DSBs for NF-κB-dependent responses of the host cell. As transcription-associated, XPG/XPF-induced DSBs had previously been shown to amplify nuclear receptor target gene expression by chromatin remodeling and loop formation as well as DNA demethylation (Le May et al., 2010, 2012), we speculated that full-scale NF-κB-dependent target gene transactivation might similarly depend on XPG/XPF-induced DSBs. This was indeed the case as determined representatively for the IL-8 and cIAP gene loci, which are optimally transcribed only in the presence of XP endonucleases. Similarly, we found that loss of XPA rendered infected cells more susceptible to apoptotic cell death, suggesting that NER activity is not only required for DSBs, but somehow simultaneously prevents the cell death that would otherwise be inevitable in the face of such massive DNA damage.

In summary, the results presented here show that the DSB induction that is a poorly understood pathogenetic trait of many bacterial pathogens is, at least in the case of the prototypical genotoxic agent *H. pylori*, a direct consequence of NF-κB and NER endonuclease activation and their coordinated recruitment to the chromatin of infected cells, a process that facilitates the full-scale expression of NF-κB target genes and likely has critical consequences for the *H. pylori*/host interaction, gastric homeostasis, and gastric carcinogenesis.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection, Bacterial Strains, Infection Conditions, and Transposon Library Screening

AGS cells (ATCC CRL 1739, a human gastric adenocarcinoma cell line) and U2OS cells (ATCC HTB96, a human osteosarcoma cell line) were grown in DMEM supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, and 100 μg/ml streptomycin. AGS cells stably expressing shRNA specific for β1-integrin have been described previously (Kwok et al., 2007). Transfection of all siRNAs was performed at 40 nM final concentration using RNAimax lipofectamine (Invitrogen; see Supplemental Experimental Procedures for siRNA sequences). DRB and ICRF-193 were purchased from Sigma-Aldrich. *H. pylori* was grown as described previously (Arnold et al., 2011) and added to cells at an MOI of 50 for 4 to 48 hr. The following previously published or newly generated strains of *H. pylori* were used: G27 wild-type, G27Δ*cag*PAI, G27Δ*cagE,* G27Δ*cagA,* P12 wild-type, P12Δ*cagL,* P12Δ*cagL*^{CagL} (Gorrell et al., 2013; Kwok et al., 2007), PMSS1, PMSS1Δ*cagE* (Arnold et al., 2011), PMSS1Δ*cagA,* PMSS1Δ*cagL,* and PMSS1Δ*cagL*^{CagL}. The PMSS1Δ*cagL* mutant was generated by replacement of the *cagL* gene with a kanamycin-resistant AphA3 cassette. For complementation, the wild-type *cagL* gene was re-introduced into the chromosomal *cagL* locus using a pSB-derived plasmid containing *cagL* with the two flanking genes (HP0538–HP0540) and chloramphenicol resistance cassette for selection. The PMSS1Δ*cagA* mutant was generated by introducing the kanamycin-resistant AphA3 cassette. Briefly, the entire cagA gene was cloned into the pGEM-T vector (Promega). The AphA3 cassette was ligated into a singular BglII site in the middle of *cagA* according to standard procedures. PMSS1 was transformed with this construct and 2 out of 20 clones were positive for CagA deletion as judged by western blotting using anti-CagA antibodies.

Bacteria in co-culture with host cells were killed with penicillin/streptomycin and 30 μg/ml tetracycline hydrochloride (Sigma). To analyze transposon mutants regarding their ability to induce DSBs in host cells, bacteria were grown on horse blood agar supplemented with 25 μg/ml chloramphenicol at 37°C for 2 days under microaerophilic conditions. Single colonies were picked, individually expanded overnight on fresh horse blood agar, and eventually inoculated in 100 μl liquid cultures (Brucella broth, Difco) containing 10% FBS (Life Technologies) in 96-well format. After 6 hr of incubation at 37°C with constant shaking, the bacteria were used for AGS cell infection in 96-well polystyrene matrix microplates (Thermo Fisher Scientific) and for cryopreservation of individual clones. For sequencing of the transposon flanking regions, the following primer was used: 5′-CAG TTC CCA ACT ATT TTG TCC-3′. All *H. pylori* liquid cultures were routinely assessed by light microscopy for contamination, morphology, and motility prior to use in infections. Immunofluorescence microscopy, fluorescence-activated cell sorting, western blotting, PFGE and chromatin enrichment procedures, qPCR, and statistics information can be found in Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental information includes Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.08.074.

AUTHOR CONTRIBUTIONS

M.L.H. performed most experiments and analyzed data; D.C.G., L.G., H.R., and K.N.K. helped with experiments; N.T. and S.B. generated numerous *H. pylori* mutants in various strains, helped perform key experiments, and provided invaluable input; and A.M. conceived the study, analyzed the data, and wrote the manuscript.

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